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2. Patent application number *(The Patent Office will fill in this part)*	**0201498.3** 23 JAN 2002
3. Full name, address and postcode of the or of each applicant *(underline all surnames)* Patents ADP number *(if you know it)*	BIOTECH RESEARCH VENTURES PTE LIMITED 11 Hospital Drive Singapore 169610 828000001
If the applicant is a corporate body, give the country/state of its incorporation	SG
4. Title of the invention	MATERIALS AND METHODS FOR TREATING CANCER
5. Name of your agent *(if you have one)*	MEWBURN ELLIS
"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	YORK HOUSE 23 KINGSWAY LONDON WC2B 6HP
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MATERIALS AND METHODS FOR TREATING CANCER

The present invention relates to materials and methods for treating cancer based on the differential gene expression in cancer cells. Particularly, but not exclusively, the present invention provides materials and methods for diagnosing and treating nasopharyngeal carcinoma.

Human nasopharyngeal carcinoma (NPC) arises in the surface epithelium of the posterior nasopharynx and associated with a high frequency of neck and distant metastases. NPC has a high incidence in certain regions of Southeast China, Southeast Asia, Taiwan, East Africa, and Alaska (Marks et al., 1998). The peak incidence for NPC occurs at the fourth to fifth decade of life. In Singapore, NPC is the fifth most prevalent cancer amongst males of Chinese descent having an annual incidence rate of 14.3 per 100,000 (Chia et al., 2000). Clinically, NPC is most commonly treated by ionizing radiation (Lee et al., 1992; Marks et al., 1998).

The World Health Organization (WHO) has classified NPC into three categories according to the degree of differentiation (Marks et al., 1998). Type I refers to

squamous cell carcinomas which are highly differentiated with characteristic epithelial growth patterns and intra- and extra-cellular keratin filaments. Non-keratinizing WHO type II carcinomas retain epithelial cell shape and growth patterns. WHO type III undifferentiated carcinomas, on the other hand, produce no keratin and have no distinctive growth pattern. WHO-I keratinizing squamous cell carcinoma comprised 75% of the U.S. nasopharyngeal carcinoma cases and were found most in U.S.-born, non-Hispanic whites. WHO-II non-keratinizing and WHO-III undifferentiated carcinomas of the nasopharynx comprised the remaining 25% of NPC and were more common in Asians. Clinically, Asians were reported to have the highest proportion of radioresponsive WHO-II nonkeratinizing and WHO-III undifferentiated carcinomas of the nasopharynx and better survival in comparison to African-Americans and Hispanic and non-Hispanic whites, who had the greatest number of the less radioresponsive kertinizing squamous cell carcinomas of the nasopharynx. The 5-year relative survival was reported to be 65% for the nonkeratinizing and undifferentiated carcinomas of the nasopharynx and 37% for the keratinizing variety (Marks et al., 1998).

Epstein-Barr virus (EBV) has been demonstrated to be closely associated with NPC (Mutirangura et al., 1998; Chen et al., 1998). The WHO type II and III NPC have been reported to be associated with EBV infection. In WHO type II and III NPC patients, they have elevated IgG and IgA levels to the EBV viral capsid antigen (VCA) as well as the diffuse component of the early antigen (Zong et al., 1992; Sigel et al., 1994). In contrast, patients with the WHO type I well-differentiated carcinomas have similar EBV serologic profiles as that of the control populations and did not appear to have a special association with EBV infection. Furthermore, molecular studies showed that EBV genomes were clearly demonstrable in the malignant epithelial tumour cells of all three WHO types of NPC. Northern blot analysis also demonstrated the expression of EBV gene products involved in the latent and lytic cycles in biopsies obtained from NPC patients (Busson et al., 1992). Nevertheless, direct evidence to show that EBV being the etiological agent for NPC has been difficult and has yet to be established.

Another important feature of NPC is that the NPC tumours are characterized histopathologically by a heavy infiltration of non-malignant lymphocytes. A significant proportion of these tumour-infiltrated lymphocytes (TILs)

have been shown to be T cells (Huang et al., 1999). The production of certain cytokines by these TILs might contribute to tumour growth during the development of NPC (Huang et al., 1999; Tang et al., 2001).

NPC carcinogenesis possibly reflects the accumulation of multiple genetic, dietary, and viral-related events that alters the normal functions of oncogenes and tumour suppressor genes (Gray and Collins, 2000; Williams, 2000). Extensive molecular analyses including karyotyping and comparative genomic hybridization (CGH) studies (Chien et al., 2001; Fang et al., 2001) have suggested that NPC arises as a multistep process. Genome-wide studies by allelotyping and CGH have detected high frequencies of genetic abnormalities on chromosomes 3p, 9p, 11q, 12q, 13q, and 14q in NPC. This data suggested the presence of a potential NPC-related tumour suppressor gene(s) to a region at 3p21.3 where the RASSF1A gene is located (Lo et al., 2001). The correlation of promoter hypermethylation with loss of RASSF1A gene expression was recently reported in NPC cells (Lo et al., 2001). Dietary exposures were found to play a role in the overall altered risk of developing specific histologic subsets of NPC. The risk of nonkeratinizing and undifferentiated tumours of the nasopharynx was increased in frequent consumers of

preserved meats containing high levels of N-nitroso compounds (Farrow et al., 1998). The risk of differentiated squamous cell carcinoma, but not other histologic types, was significantly reduced in individuals with vitamin C intake (Farrow et al., 1998). This association was markedly stronger among non-smokers and former smokers than among current smokers (Farrow et al., 1998). Furthermore, titres of EBV antibody to early antigen and viral capsid antigen were found to be elevated in patients with non-keratinizing and undifferentiated carcinomas of the nasopharynx whereas the titers of these antibodies are comparable between the controls and patients with the keratinizing variety (Neel, 1985 and 1986). However, the molecular basis between the kertinizing squamous cell carcinomas of the nasopharynx and the relatively more radioresponsive nonkeratinizing and undifferentiated carcinomas of the nasopharynx have not been studied systematically.

To try to understand the molecular differences between kertinizing squamous cell carcinomas of the nasopharynx and the nonkeratinizing and undifferentiated carcinomas of the nasopharynx, the present inventors have employed cDNA microarrays to identify genes that might potentially be involved in the carcinogenesis of human NPC. The

inventors have determined a small number of genes that are differentially expressed in undifferentiated and differentiated human NPC. Specifically, the inventors have found that fifteen genes were differentially up-regulated in the undifferentiated CNE-2 NPC cells, while six gene were specifically up-regulated in the well differentiated HK1 cells.

One of the genes identified to be specifically up-regulated in the undifferentiated human NPC cell line CNE-2 is the human imprinting gene H19. Interestingly, H19 is not expressed in the well-differentiated human HK1 NPC cells. Northern blot and *in situ* hybridization analyses also confirmed that the H19 gene is strongly expressed in the undifferentiated CNE-2 human NPC cell line but not in the well-differentiated HK1 human NPC cell line. Furthermore, the inventors have demonstrated that de-regulation of the H19 gene expression in the well-differentiated human HK1 NPC cells could be induced by the hypomethylation of CpG sites of the H19 promoter region. The inventors believe that hypermethylation of gene promoter regions may therefore be an important epigenetic event that plays a role in the differentiation of human NPC cells and the transcriptional silencing of imprinted genes.

Thus, at its most general, the present invention provides materials and methods for diagnosing and treating nasopharyngeal carcinoma (NPC). The invention further provides methods of screening for agents or therapeutic targets that may be used in the treatment or diagnosis of nasopharyngeal carcinoma.

Knowledge of the differential expression of certain genes in the different types of NPC, provides for the first time a tool for diagnosing NPC or a risk of NPC. This diagnosis may be independent of histology studies or may be used to complement histology studies.

Further and very importantly, the knowledge of differential gene expression enables diagnosis of the type of NPC, thus ensuring that the appropriate treatment is given.

In a first aspect of the present invention, there is provided a method for determining the presence or risk of a NPC in a patient comprising the steps of

(a) obtaining expression products from a nasopharyngeal cell obtained from a patient suspected of having or at risk of having a NPC;

(b) contacting said expression products with a binding members capable of binding to expression products corresponding to one or more genes identified in Table 1; and

(c) determining the presence or risk of NPC in said patient based on the binding of the expression products from said nasopharyngeal cell to the one or more binding members.

The presence or up-regulation of an expression product may be determined by comparing the presence or level of the expression product obtained from the cell under test with those from an appropriate control cell. Ideally, the control cell would be a "normal", i.e. non-cancerous epithelial cell from the nasopharynx. These cells could also be obtained from the patient under examination. Normal epithelial cells from other parts of the body could also be used. An alternative to the analysis of a control cell is the production of expression standards that could be used as a control to compare with the expression level or pattern from the cell under test. Such standards may be produced by analysing a collection of samples to determine a "standard" expression level or pattern of one or more products in normal cells. This is discussed in more detail below.

As mentioned above, the method according to the first aspect of the invention is not only particularly suited for classifying a nasopharyngeal sample as normal or malignant, but also classifying the particular type of NPC.

Thus, in one embodiment, the invention provides a method for determining the type of NPC, e.g. differentiated or undifferentiated by detecting the differentially up-regulated expression of at least one gene identified in Table 1.

The expression product may be a transcribed nucleic acid sequence or the expressed polypeptide. The transcribed nucleic acid sequence may be RNA, mRNA or cDNA produced from mRNA.

The binding member may a complementary nucleic acid sequence which is capable of specifically binding to the transcribed nucleic acid under suitable hybridisation conditions.

Where the expression product is the expressed protein, the binding member is preferably an antibody or a molecule

comprising an antibody binding domain specific for said expressed polypeptide.

The binding member may be labelled for detection purposes using standard procedures known in the art.

Preferably, the binding member is fixed to a solid support. The expression products may then be passed over the solid support, thereby bringing them into contact with the binding member.

The present inventors have successfully used a nucleic acid microarray comprising a plurality of nucleic acid sequences fixed to a solid support. By passing nucleic acid sequences representing expressed genes, over the microarray, they were able to create an expression profile characteristic of NPC and furthermore, the type of NPC.

In a second aspect of the present invention, there is provided a method of creating an expression profile characteristic of NPC or a particular type of NPC, said method comprising

(a) obtaining expression products from a NPC cell obtained from a patient

(b) contacting said expression products with a plurality of binding members capable of specifically binding to expression products of one or more genes identified in Table 1;

(c) determining the binding of said expression products with the binding members so as to create an expression profile characteristic of the NPC cell.

The invention further provides a nucleic acid (RNA or cDNA) expression profile database comprising expression data characteristic of a NPC or type of NPC, said data being obtained from analysis of a plurality of oligonucleotide microarrays showing nucleic acid distribution characteristic of NPC or a type of NPC, for use in diagnosis.

The present invention further provides an oligonucleotide microarray for diagnosing a NPC or type of NPC, said microarray comprising a solid support housing a plurality of oligonucleotide sequences, said oligonucleotides individually comprising nucleic acid sequence capable of specifically binding to expressed nucleic acid of a plurality of genes identified in Table 1.

In a third aspect of the present invention, there is provided a kit for determining the presence or type of NPC in a biological sample, said kit comprising a one or more binding members capable of specifically binding to an expression product of one or more genes identified in Table 1, and a detection means. The biological sample is preferably cell extract.

Preferably, the one or more binding members (antibody binding domains or nucleic acid sequences) in the kit is fixed to a solid support. The detection means is preferably a label (radioactive or dye e.g. fluorescent dye) that detects when a binding member has bound to an expression product.

Preferably, the one or more binding members include a binding member capable of specifically binding to an expression product of H19 or CDKNIC. Both of these genes serve as convenient markers for undifferentiated. As H19 does not produce a protein product, the expression product will be mRNA. In the case of CDKNIC, the expression product can be mRNA or the resulting protein product.

As mentioned above, type II and type III undifferentiated NPC are more responsive to radiotherapy and consequently

there is a better survival rate in patients suffering from these types of NPC. The present inventors have determined a number of genes that are up-regulated in undifferentiated NPC as opposed to differentiated, type I NPC (see Table 1). These genes include H19 and CDKN1C.

The inventors have further determined a number of genes that are up-regulated in type I differentiated cells as opposed to undifferentiated (type II or type III) cells (see Table 1).

Not only does the knowledge of this differential expression lead to extremely useful diagnostic methods, but it also provides new approaches in the treatment of NPC, particularly Type I which has had limited treatment success in the past.

The inventors have surprisingly found that the promoter region of the H19 gene is highly methylated in differentiated cells whereas no methylation is seen in the same region in undifferentiated cells. The inventors have further shown that demethylation of this region leads to the expression of the H19 gene in differentiated cells.

This exciting discovery provides a way to change the differential expression of genes characteristic of different types of NPC and render the cells more susceptible to treatment, e.g. radiotherapy.

Thus, in a fourth aspect of the invention there is provided a method of treating a patient with or at risk from NPC comprising administering a demethylation agent, e.g. 5'aza-2'-deoxycytidine, in association with a cancer treatment, e.g. chemo or radiotherapy.

The invention also provides the use of a demethylation agent for preparing a medicament for treating nasopharyngeal carcinoma in association with chemo or radiotherapy.

It is preferred that the demethylation agent is used in the treatment of type I NPC.

The inventors' findings that differentiated and undifferentiated forms of NPC have different gene expression, enables the development of a method for screening for substances capable of treating NPC and particularly, substances capable of selectively treating different types of NPC.

Thus, in a fifth aspect, there is provided a method of screening for substances capable of treating NPC in a patient, said method comprising

(a) over-expressing in a cell one or more genes identified in Table 1,

(b) contacting said cell with a test substance;

(c) determining the effect of said test substance on said cell as compared to the effect of said test substance on a comparable cell absent of the over-expression of said one or more genes; and

(d) identifying said test substance as a substance capable of treating NPC.

The method may further comprise the step of producing a pharmaceutical composition comprising the substance identified in step (d).

The one or more genes may be over-expressed by inserting into said cell nucleic acid capable of expressing expression products characteristic of said genes.

Depending on the screening method, it may be preferably to choose genes known to be up-regulated in either differentiated NPC or undifferentiated NPC. Further,

depending on the substance under test, it may be preferable to choose those genes known in produce a protein product, e.g. CDKNIC.

In a preferred embodiment, the one or more genes being expressed include CDKN1C.

The method may also include the treatment of the cell over-expressing the one or more genes identified in Table 1 with a demethylation agent in association with the test substance.

As an alternative to recombinantly producing a cell over-expressing one or more genes characteristic of the different types of NPC, a NPC cell (Type I, II or III) could be used directly. Although this would provide valuable information concerning the effect of the test substance, further tests may be needed to identify the specific gene target.

Aspects and embodiments of the present invention will now be illustrated, by way of example, with reference to the accompanying figures. Further aspects and embodiments will be apparent to those skilled in the art. All

documents mentioned in this text are incorporated herein by reference.

In the figures:

Figure 1

Comparative microarray analyses of gene expression between undifferentiated NPC (CNE-2) cells and well-differentiated NPC (HK1) cells, from three experiments (1 to 3) performed in duplicates. cDNAs derived from CNE-2 cells or HK1 cells were labelled with Cy5 (pseudo-colored red on scanning) and reference cDNA (pooled from 10 cell lines) with Cy3 (pseudo-colored green). Log_2-transformed median-centered Cy5:Cy3 ratios are calculated using the Cluster program. These ratios are a measure of relative gene expression in each experimental sample and are displayed as a spectrum of graded colors from red through black to green, according to the color bar shown at the bottom (using the TreeView program). Red represents a Cy5:Cy3 ratio that is higher than the median for a particular gene across experimental samples. Green or black represents a Cy5:Cy3 ratio that is lower than or equivalent to the median for the gene across experimental samples, respectively. Four gene clusters (A to D) are represented, showing: (A) genes that are expressed at

higher levels in HK1 than in CNE-2 cells, (B and C) genes that are expressed at higher levels in CNE-2 cells than in HK1, and (D) internal 'house-keeping' control genes.

Figure 2

Northern blot analysis of polyA^{+} RNA purified from 18 different human tumor cell lines (Detroit 562, Fadu, CNE-2, DAKIKI, Raji, WT-18, FHS-738Lu, MRC-5, A549, HeLa, HT-3, SW480, PA-1, HeCat, Bt-20, Hep-G2, A498 and Hs67). 5µg of polyA^{+} RNA was subjected to electrophoresis in 1% formaldehyde-containing agarose gel, transferred to nylon membrane and probed for H19 and β-actin as described in 'Materials and Methods'.

Figure 3

In situ hybridization of cell lines derived from undifferentiated NPC (CNE-2), well-differentiated NPC (HK1) and cervical carcinoma (HT-3) using β-actin probe and H19 sense and anti-sense probes as described in 'Materials and Methods'. Photographs were taken at magnification x400.

Figure 4

In situ hybridization of primary human tissues from normal nasopharynx (NP) and undifferentiated carcinoma of

the nasopharynx (NPC). DIG-labelled probes specific for H19 or β-actin were used as described in 'Materials and Methods'. Photographs were taken at magnification x400.

Figure 5

Northern blot analysis of polyA$^+$ RNA from (A) sixteen adult tissues (heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon and leukocyte) and (B) five fetal tissues (heart, brain, lung, liver, kidney) on nylon membrane (MTN Blots, Clontech). Blots were probed for H19 and β-actin as described in 'Materials and Methods'.

Figure 6

Northern blot analysis of RNA from human cell lines, CNE-2 and HK1, derived from undifferentiated and well-differentiated carcinomas of the nasopharynx respectively, using probes for H19, insulin-like growth factor 2 (IGF-2) and β-actin.

Figure 7

The extent of CpG methylation in the 304-bp region within H19 promoter was examined using bisulphite sequencing. The methylation profile of the twelve CpG sites of this

region were analysed in undifferentiated NPC cells (CNE-2), well-differentiated NPC cells (HK1) and HK1 cells treated with 5'-aza-2'-deoxycytidine (AzC), an inhibitor of methylation. The occurrence of methylation at each CpG site is expressed as a percentage of the number of clones sequenced. The number of sequenced clones derived from CNE-2, HK1 and AzC-treated HK1 cells were 19, 63 and 27 respectively.

Figure 8

Northern blot analysis to examine H19 gene expression in well-differentiated NPC (HK1) and undifferentiated NPC (CNE-2) cells, cultured for 7 days in the presence or absence of 5'-aza-2'-deoxycytidine (AzC). Blot was probed for H19 and β-actin as described in 'Materials and Methods'.

MATERIALS AND METHODS

Cell lines and tissue culture

The human NPC cell lines CNE-2, and HK1 had been described previously (Sizhong et al., 1983; Huang et al., 1983). The CNE-2 cells were obtained from Professor H. M. Wang (Cancer Institute, Sun Yat-sen University of Medical Sciences, Guangzhou, People's Republic of China), while the cell line HK1 was obtained from Professor D. P. Huang

(The Chinese University of Hong Kong). CNE-2 cells are derived from undifferentiated nasopharyngeal carcinoma (Sizhong et al., 1983), while HK1 was derived from patient with the well-differentiated squamous carcinoma of the nasopharynx (Huang et al., 1983).

Most of the tumor cell lines employed in the present study were obtained from the American Tissue Type Collection (ATCC) unless otherwise stated. These human cell lines include A498 (kidney carcinoma), A549 (lung carcinoma), DAKIKI (EBV-transformed lymphoblast), Fadu (pharyngeal carcinoma), HeLa (cervical adenocarcinoma), HepG2 (heptocellular carcinoma), MCF-7 (breast adenocarcinoma), HT-3 (cervical carcinoma), K562 (myeloid leukaemia), Detroit-562 (pharynx carcinoma), Raji (Burkitt lymphoma), WT-18 (EBV-transformed B-lymphocyte), FHS-738Lu (normal lung), MRC-5 (diploid lung). Additional cell lines employed include the SW480 (colon adenocarcinoma), PA-1 (ovarian teratocarcinoma), HeCat (epithelial), BT-20 (breast carcinoma) and Hs67 (normal thymus). All these cell lines were propagated in RPMI medium (Gibco BRL, Life Technologies, Grand Island, NY) supplemented with 10% FCS (Hyclone, Logan, UT), 0.1 mM non-essential amino acids, 4mM L-glutamine, and 1mM sodium pyruvate.

Tissue specimens

Human NPC tumor biopsies were obtained prior to treatment from patients with informed consents at the Department of ENT of the Singapore General Hospital. Biopsies were obtained from patients under topical anaesthesia using 4% cocaine solution applied with a cotton swab applicator. A total of three bites of tumour tissues were taken using Hilyard forceps under direct vision with a fibre-optic naso-endoscope. The first two bites were sent for histological examination and the third biopsy obtained was taken for the present study. Tumour biopsies taken from patients were immediately snap-frozen and stored in liquid nitrogen until being studied. Histo-pathological diagnosis was confirmed in paraffin sections.

cDNA microarrays

The inventors have selected over 1000 IMAGE human cDNA clones (Incyte Genomics Inc., Palo Alto, CA), representing approximately 941 distinct Unigene clusters (i.e. unique genes), for their spotted microarray studies. These 1000 clones form part of a pool of 18,000 clones established as a core facility for cDNA microarray analyses at the National Cancer Centre, Singapore. The full listing of these clones will be made available on request. These

1000 clones were streaked out and individual colonies grown overnight. Of these, 713 clones were correctly identified and verified by PCR amplification using gene-specific primer pairs. Each of the inserts was amplified from an overnight bacterial culture, using a final dilution of 1:1000 in a 100 μl PCR reaction. The PCR products were concentrated, resuspended in 20 μl of 3XSSC and then employed for printing on poly-L-lysine (Sigma Diagnostics, St. Louis, MO)-treated glass microscope slides (Fisher) using a robotic GMS 417 microarrayer (Genetic Microsystems Inc, Woburn, MA) fitted with four printing ring-pins (TeleChem International Inc, Sunnyvale, CA). Housekeeping genes including GAPDH, β-actin, β-2-microglobulin, cyclophilin and ubiquitin were similarly spotted as internal controls for the normalization of hybridization signals during data analysis. Following printing, the slides were inverted over a boiling water-bath (reagent grade water) for 2-3 seconds to rehydrate the array, snap-dried for 5 seconds on a 100°C heating block for 4 seconds and cross-linked with 550 mJ ultraviolet irradiation using a Stratalinker (Strategene, La Jolla, CA). The slides were then placed in 0.2% SDS (10 minutes, with magnetic stirrer), followed by 5 washes in clean water (2L) before transferring to boiling-hot water (10 minutes), blotted to remove excess liquid,

desiccated for 5 minutes in 95% ethanol and air-dried for 5 minutes in an 80°C oven.

cDNA microarray hybridization

The protocol accompanying the 3DNA Expression Array Detection Kit (Genisphere Inc., Montvale, NJ) for the synthesis of hybridization probes was used, with modifications. cDNA was synthesised by reverse transcription using 10 µg of total RNA extracted from human NPC cells or from 10 µg of reference RNA (pooled from 10 cell lines) with oligo(dT) primers incorporating either the capture sequence for the 3DNA Cy5 'labelling' reagent (5′- CCTGTTG CTCTATTTCCCGTGCCGCTCCGGT-(dT)$_n$-3′) or the 3DNA Cy3 'labelling' reagent (5′GGCCGACTCACTGCGCGTCTTCTGTCCCGCC-(dT)$_n$-3′), respectively. The 10 cell lines from which the pooled reference RNA was generated were A498, A549, DAKIKI, CNE-2, Fadu, HeLa, HepG2, MCF-7, HT-3, and K562. cDNAs generated from each of the test RNA samples (CNE-2 or HK1) as well as the reference RNA were competitively hybridized to the microarray using a hybridization volume of 20 µl under a glass coverslip and in a dark humidified chamber (TeleChem International Inc, Sunnyvale, CA) overnight at 42°C.

Post-hybridisation slide washes involve a series of washes, starting with 2x SSC / 0.1% SDS (2 washes, 5 minutes each), followed by 0.2x SSC / 0.1% SDS (2 washes, 5 minutes each), and finally with 0.1x SSC (2 washes, 5 minutes each). The cDNA, which incorporates a fluorescent dye capture sequence, is labelled with Cy5 or Cy3 only after the cDNA has hybridised to the microarray and the excess unbound cDNA washed off.

Quantitation of arrays and cluster analysis

Hybridized arrays were scanned with a GMS 418 laser scanner (Genetic Microsystems Inc, Woburn, MA). Images for Cy5 and Cy3 were acquired separately using different channels, superimposed and quantified with Imagene software version 3.0 (BioDiscovery Inc, Los Angeles, CA). Spots on the array were defined by aligning a grid of circles over each spot on the entire array image. The net signal for each spot was obtained by subtracting the background signal from the average intensity within the spot. The signal intensities obtained from both Cy5 and Cy3 channels were normalized by applying a scaling factor such that the mean Cy5:Cy3 ratio of spots across the entire array is 1.0. Log_2-transformation and centering of the median for the Cy5:Cy3 ratio were then computed. A hierarchical clustering algorithm was applied using

complete linkage clustering (Gene Cluster program, http://rana.lbl.gov/; Eisen et al., 1998). The TreeView program (Eisen et al., 1998) was used to visualize the clustered data by displaying the intensity of gene expression using a spectrum of graded colors from bright red, through black, to bright green.

Northern blot analysis

Total cellular RNA was isolated using TRIzol Reagent (Gibco BRL, Life Technologies, Grand Island, NY). Poly(A)$^+$ RNA was selected by using the Fast-Track mRNA isolation kit from Invitrogen (Invitrogen Corp., San Diego, CA). For Northern blotting analysis, polyA$^+$ RNA (5 μg) was loaded in each lane of a 1% agarose gel containing 0.7% formaldehyde and 5mM iodoacetamide, and subjected to electrophoresis. RNA was transferred to Hybond-N$^+$ nylon membrane (Amersham, Piscataway, NJ) by capillary transfer and probed with ^{32}P-labelled H19 DNA (full-length cDNA clone obtained from Professor Shirley Tilghman, Princeton University, NJ). Probes were labelled by random hexa-nucleotide priming using the High Prime DNA labelling kit (Boehringer Mannheim GmbH, Mannheim, Germany) according to manufacturer's protocol. The filters employed for the human and human fetal multiple tissue Northern blot were purchased from Clontech Laboratories (Clontech

Laboratories Inc., Palo Alto, CA). Hybridization signals were quantitated using the BioRadFX PhosphorImager (BioRad, Richmond, CA).

In situ hybridization

Frozen biopsy NPC tissues were sectioned to 10 µm in a cryostat. Cell-lines (CNE-2, HK1 and HT-3) were grown to half confluence in chambers mounted on glass slides (Falcon CultureSlide, Becton Dickinson and Co., NJ). Hybridizations were performed with non-radioactive sense and anti-sense H19 probe, which was labelled by the incorporation of digoxigenin (DIG)-labeled dUTP (DIG RNA Labelling Kit, Hoffmann-La Roche, Basel, Switzerland), according to manufacturer's instructions. The hybridized digoxigenin-labeled probes were detected with a peroxidase-conjugated anti-DIG antibody and subsequent enzyme-catalyzed color reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium salt (Boehringer Mannheim GmbH, Mannheim, Germany). Sections were counter-stained with haematoxylin (BDH Laboratory Supplies, Dorset, England). Slides were viewed with the Olympus BX51 microscope (Olympus Optical Co. Ltd., Tokyo, Japan).

Treatment with 5′-aza-2′-deoxycytidine

Seven cell lines (CNE-2, HK1, HeLa, Hep-G2, HT-3, NIH:OVCAR-3 and SW480) were cultured separately for 7 days in RPMI (containing 10% fetal bovine serum) in the presence or absence of 12.5 μM 5′-aza-2′-deoxycytidine (Sigma Diagnostics, St. Louis, MO). Total RNA from these cell lines was extracted using TRIzol Reagent (Gibco BRL, Life Technologies, Grand Island, NY), according to manufacturer's instruction. Twenty μg of total RNA was used for Northern blot analysis.

Bisulphite sequencing of the H19 promoter region

Genomic DNA (2 μg) was digested with RsaI at 37°C for 16h and denatured by adding freshly prepared NaOH to a final concentration of 0.3M at 42°C for 30min. The bisulphite reaction was carried out on the denatured DNA by adding urea/bisulphite solution and hydroquinone to final concentrations of 5.36M, 3.44M and 0.5mM respectively. The reaction involves 20 cycles of 55°C (15 minutes) followed by denaturation at 95°C (30 seconds). The bisulphite-treated DNA (5 μl) was amplified by PCR in a 20 μl reaction with 0.5 units of AmpliTaq DNA polymerase (Perkin-Elmer Corp., Norwalk, CT) and using primers (10 μM) that will amplify a 306-bp region in the H19

promoter: 5′-AGATAGTGG TTTGGGAGGGAGAGGTTTTGGAT-3′ and 5′-ATCCCACCCCCTCCCTCACCCTACT CCTCA-3′. The reaction was subjected to 94°C (3 minutes), then 35 cycles (of 94°C for 30 seconds, 58°C for 1 minute, 72°C for 30 seconds), and ending with 72°C (6 minutes). The bisulphite-treated DNA was then cloned and sequenced as described (Tremblay et al., 1997). DNA sequencing was carried out using a CEQ 2000 capillary sequencer (Beckman Coulter Inc., Fullerton, CA).

RESULTS

The undifferentiated human NPC cell line CNE-2 and the well-differentiated human HK1 NPC tumour cells demonstrated unique gene expression profile

To identify human NPC-specific genes, the inventors have initiated a program at the National Cancer Centre, Singapore, to employ a library comprising 18,000 cDNA clones for the screening of human nasopharyngeal carcinoma clinical biopsies to link these expression profiles in the context of clinical information. Based on their preliminary data, they have chosen approximately 1000 genes for their present study.

Gene expression profiles were established using RNA extracted from the undifferentiated human NPC cell line CNE-2 and the well-differentiated NPC cell line HK1 and hybridized to spotted microarrays. CNE-2 and HK1 cells exhibited distinct gene expression profiles (Figure 1, Table 1). Six genes out of the approximately 1000 genes studied were found to be consistently up-regulated in the HK1 cells in comparison to the CNE-2 cells (Table 1). These include the genes that encode metallothionein-I, human melanoma-associated antigen B3, and monocyte chemotactic protein-3 (MCP-3) (Figure 1A, Table 1). In comparison, there are fifteen genes that were found consistently to be more highly expressed in the RNA of the undifferentiated CNE-2 cells than that of the well-differentiated HK1 cells (Table 1). Some of these genes include the H19 imprinted gene, the cyclin-dependent kinase inhibitor 1C (CDKN1C or $p57^{KIP2}$) gene, genes that encode protein-tyrosine kinase Flt4, Tat-interacting protein, and cyclin D3 (Figure 1B and C, Table 1).

H19 gene is highly expressed in undifferentiated human NPC cells

The specific up-regulation of the imprinted H19 gene in the undifferentiated CNE-2 NPC cells is most interesting.

To examine whether the expression of H19 is unique to human NPC cells, the inventors performed Northern blot analysis to compare to expression of H19 in eighteen different human tumour cell lines of diverse origins. These include tumour cell lines that were derived from human Burkitt lymphoma, pharyngeal carcinoma, cervical carcinoma, lung carcinoma, colorectal carcinoma, ovarian teratocarcinoma, hepatocellular carcinoma, kidney carcinoma, breast carcinoma, EBV-transformed normal B lymphocytes, fibroblast, epithelium and the thymus (Figure 2). Positive hybridization with the H19 probe could only be detected for the CNE-2 cells (Figure 2). The other seventeen cell lines tested under these conditions did not have detectable H19 gene expression.

The specific expression of H19 in the human undifferentiated CNE-2 NPC cell line was also confirmed by *in situ* hybridization studies (Figure 3). Although the expression of β-actin could be detected in the CNE-2, HK1, and HT-3 (cervical carcinoma) cells tested, the expression of H19 could only be specifically detected in CNE-2 cells (Figure 3). The H19 mRNA expressing cells were identified by the grey-brown color staining following binding to the non-radioactive, digoxigenin-labelled anti-sense H19 RNA probe (Figure 3).

To address the relevance of the expression of H19 in the undifferentiated CNE-2 cells, the expression of H19 in undifferentiated human primary NPC tissues by *in situ* hybridization studies was performed. *In situ* hybridization studies revealed that H19 also expressed ~~strongly in undifferentiated human NPC biopsies (Figure 4)~~ and not in the epithelium of chronic inflammatory tissue biopsies that were negative for malignancy but were taken similar conditions from the nasopharyngeal region (Figure 4). A total of seven undifferentiated human primary NPC biopsies and three non-NPC biopsies were studied by *in situ* hybridization and representative results were shown in Figure 4.

Furthermore, it was also determined, by Northern blot analysis, that H19 is expressed in human placenta tissues (Figure 5A). H19 could not be detected in RNA derived from most of the adult tissues tested. These included tissues of the heart, brain, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon and leukocyte (Figure 5A). The expression of H19 could also be detected in the RNA of fetal liver but not tissues of the

fetal heart, fetal brain, fetal lung nor the fetal kidney (Figure 5B).

H19 is a paternally imprinted gene and is located in close proximity to the maternally imprinted IGF-2 gene on chromosome 11p15.5 (Feinberg, 1999). To determine if there is a relationship between the expression of these two genes in the undifferentiated CNE-2 cells and the well-differentiated HK1 cells, Northern blot analyses were performed. In contrast to H19 that is only expressed in CNE-2 cells, IGF-2 is expressed in both CNE-2 and HK1 cells (Figure 6).

The CpG dinucleotides in the promoter region of the well differentiated HK1 NPC cells are hypermethylated

When the DNA sequence of the H19 was examined, one noticeable feature is the presence of many CpG dinucleotides in the promoter region of the H19 gene (Figure 7). The hypo- and hyper-methylation of CpG dinucleotides have been demonstrated to be an important epigenetic event in the regulation of gene transcription. To investigate whether methylation plays a role in the regulation of the H19 gene in undifferentiated and well-differentiated NPC cells, the inventors compared the

promoter methylation status of H19 gene in CNE-2 and HK1 cells. Genomic DNA were purified from CNE-2 cells and HK1 cells and the methylation status of the H19 promoter was assessed following PCR amplification of the H19 promoter region and bisulfite sequencing. Sodium bisulphite induces specifically the hydrolytic deamination of cytosine residues and not 5-methylcytosine residues. Therefore, it is expected that when the PCR-amplified DNA were sequenced following bisulphite treatment, the cytosines detected in the final sequencing reactions will represent those cytosine residues that were methylated in the native DNA sample. In comparison, all cytosine residues that were not methylated in the original DNA sample will subsequently be converted to thymine following the bisulphite treatment. A total of twelve CpG dinucleotides spanning 304bp of the H19 promoter region were studied (Figure 7). Most of these CpG dinucleotides were not methylated in the DNA purified from the CNE-2 cells of which H19 is strongly expressed (Figure 7). In comparison, most of these CpG dinucleotides were methylated in the genomic DNA purified from the HK1 cells of which H19 is not expressed (Figure 7). The CpG dinucleotides at positions -209, -189, -180, -117, and -102 appear to be methylation "hot-spots" and accounted for greater than 70% of the clones sequenced (Figure 7).

Hypomethylation of the CpG dinucleotides within the H19 promoter region correlated with the restoration of H19 gene expression in the well-differentiated HK1 NPC cells

To determine whether the expression of the H19 gene could be induced by hypomethylation, the inventors have treated the HK1 cells with the demethylating agent 5′-aza-2′-deoxycytidine. When the RNA extracted from HK1 cells following treatment with the demethylating agent 5′-aza-2′-deoxycytidine were analyzed by Northern blot hybridization with the H19 probe, abundant amount of the H19 transcript could be detected in the RNA of the treated HK1 cells (Figure 8).

To further address the relevance of promoter hypomethylation and the expression of the H19 gene in the HK1 cells, genomic DNA were purified from the HK1 cells following treatment with the demethylating agent 5′-aza-2′-deoxycytidine and employed for bisulfite sequencing as described above. In contrast to the CpG dinucleotides that are mostly methylated in the DNA purified from the wild type HK1 cells, the CpG dinucleotides within the H19 promoter region of the DNA purified from the 5′-aza-2′-deoxycytidine-treated HK1 cells are much less methylated

(Figure 7). These findings suggest that hypomethylation of the H19 promoter region is correlated with the expression of H19 gene in human NPC cells.

DISCUSSION

Human NPC are classified into Types I, II, and III according to their degrees of differentiation and keratinization (Marks et al., 1998). Type I is the squamous cell NPC carcinomas that are highly differentiated and relatively less radioresponsive. Type III undifferentiated NPC carcinomas, on the other hand, are more radioresponsive (Neel 1985; Marks et al., 1998). The molecular mechanism for tumor promotion and progression in human NPC is, at best, partially understood and there is no study on the relationship of the differentiation status of NPC cells and carcinogenesis. Genetic alterations have been implicated as one of many mechanisms likely to contribute towards the development of NPC. Most of these genetic alterations will be reflected by a subsequent change in the respective gene products. In Singapore, it has been suggested that more than 90% of clinically detected NPC cases are poorly differentiated. In this study, the inventors have therefore employed cDNA microarrays to

identify genes whose expression differs in well-differentiated and undifferentiated NPC carcinoma cells. These genes will undoubtedly be important for elucidating human NPC carcinogenesis. From their cDNA microarray analyses, fifteen genes were demonstrated to be differentially upregulated in the undifferentiated CNE-2 NPC cells, while six genes were specifically upregulated in the well-differentiated HK1 cells (Figure 1 and Table 1).

One of the genes that is consistently upregulated in the well-differentiated HK1 cells is metallothionein I (Figure 1 and Table 1). Metallothionein I encodes a metal-binding protein that functions in cell growth, repair and differentiation, and has been implicated to be a potential marker for tumour differentiation or cell proliferation (Hengstler et al., 2001). Furthermore, metallothionein I also plays a protective role against DNA damage and apoptosis induced by oxidative or external stress, and has postulated to contribute towards radiation resistance in tumour cells (Jayasurya et al., 2000). Other genes that were also differentially up-regulated in HK1 cells include those encoding the monocyte chemotactic protein-3 (MCP-3), CPR2, CDK inhibitor 2A and IGFBP-3 (Figure 1 and Table 1).

MCP-3, a C-C chemokine that interacts with chemokine receptors CCR1, CCR2, and CCR3, and is a chemo-attractant for monocytes, T cells, NK cells, eosinophils, and dendritic cells (Fioretti et al., 1998). It has been suggested that the characteristic leukocyte infiltration seen in NPC tumour lesions might be induced by C-C chemokines secreted by the infiltrating cells (Tang et al., 2001). However, the up-regulation of MCP-3 expression in the HK1 NPC cells suggested that the NPC tumor cells themselves could also contribute actively in recruiting lymphocytes to the tumour site.

Fifteen genes were found to be consistently differentially expressed at higher levels in the undifferentiated CNE-2 cells in comparison to the well-differentiated HK1 NPC cells (Figure 1 and Table 1). One of these genes encodes protein-tyrosine kinase Flt4, a receptor-type tyrosine kinase, with which angiogenic vascular endothelial growth factor-C (VEGF-C) interacts (Lee et al., 1996). Interestingly, the enhanced expression of Flt4 in undifferentiated NPC cells concurs well with the observation that Flt4 is expressed in nondifferentiated teratocarcinoma cells but not expressed in differentiated teratocarcinoma cells (Pajusola et al.,

1992). Another gene that is up-regulated in CNE-2 cells is the gene that encodes the Tat-interacting protein 30kDa (TIP30). TIP30 is identical to CC3 that function as a suppressor of metastasis and inhibits the metastasis of human small cell lung carcinoma by promoting tumour cells to undergo apoptosis (Shtivelman 1997). This is mediated by the induction of a number of apoptosis-related genes such as Bad and Siva, and the metastasis suppressor, NM23-H2 by TIP30/CC3 (Xiao et al., 2000).

Interestingly, it was demonstrated that the H19 gene and the gene encoding CDKN1C were differentially up-regulated in the undifferentiated CNE-2 NPC cells (Figure 1 and Table 1). Both H19 and CDKN1C genes are located at chromosome 11p15 (Feinberg, 1999) and both are reported to be imprinted genes. Genomic imprinting is a parental origin-specific chromosomal modification that causes differential expression of maternal and parental genes (Tilghman 1999). Although a relatively small number of genes has been reported to be imprinted, they nevertheless play important roles in development and carcinogenesis (Joyce and Schofield, 1998). Both the CDKN1C and H19 genes have been postulated to be tumor-suppressor genes (Hatada and Mukai, 1995). It has also been demonstrated that CDKN1C is a potent inhibitor of

many G1 cyclin/Cdk complexes and a negative regulator of cell proliferation (Matsuoka et al., 1995; Hatada et al., 1996 & 1995).

H19 is a paternally imprinted gene with unknown function. It is located in close proximity to the maternally ~~imprinted IGF-2 gene on chromosome 11p15.5 (Feinberg~~ 1999). For normal human tissues, expression of H19 could be detected in the placenta and fetal liver tissues tested but not expressed in the other adult and fetal tissues (Figure 5). This concurs well with studies in mouse, where the H19 gene is highly expressed in endoderm and mesoderm tissue of mouse embryos, but is dramatically down-regulated after birth (Brunkow and Tilghman, 1991).

At present, the function of H19 gene in carcinogenesis is unclear. However, the over-expression of the H19 gene in transgenic mice caused prenatal lethality in the late portion of the gestational period, strongly suggest, but does not prove, an important role for H19 during development and differentiation (Brunkow and Tilghman, 1991; Pfeifer et al., 1996). Consistent with these observations, it has been reported that the H19 gene is re-expressed in rat vascular smooth muscle cells after injury (Kim et al., 1994). There have also been a number

of indications that genomic imprinting may be important in human disease (Paulsen et al., 2001). It has been reported that some patients with Beckwith-Wiedemann syndrome show uniparental disomy at 11p15 (Bliek et al., 2001). Several studies have further demonstrated the preferential retention of paternal alleles in embryonal tumours such as the Wilms' tumour (Moulton et al., 1994; Taniguchi et al., 1995) and embryonal rhabdomyosarcoma (Casola et al., 1997; Zhan et al., 1994) that had undergone loss of heterozygosity at tumour suppressor gene loci. These observations supported nonequivalence of the two alleles and suggesting a possible role for genomic imprinting in tumorigenesis (Zhang et al., 1993). It has also been shown that normal imprinting is relaxed, and gene expression is biallelic in a majority of Wilms' tumours that retain heterozygosity at this locus (Moulton et al., 1994; Taniguchi et al., 1995). The tumour-suppressor potential of the human H19 gene has also been demonstrated. Transfection of the H19 gene into two embryonal tumour cell lines abrogated the oncogenicity of some of the transformed cells in soft agar and their tumorigenicity in nude mice (Hao et al., 1993).

When the gene expression pattern of the H19 gene was examined and compared between well-differentiated and

undifferentiated human NPC cells, it was demonstrated that H19 gene expression could only be specifically demonstrated in the undifferentaited CNE-2 human NPC cells (Figures 2, 4 and 6). This was also confirmed for human NPC biopsy tissues where H19 was expressed in undifferentiated NPC cells and not in the epithelium of ~~normal nasopharyngeal (NP) tissues (Figure 4). It is~~ interesting to observe that the expression of the H19 gene differs for the two NPC cell lines that exhibited different degree of differentiation. More importantly, we demonstrated that the expression of H19 could be reversed by culturing the well-differentiated HK1 cells in the presence of 5′-aza-2′-deoxycytidine (Figure 8). Furthermore, the expression of H19 correlated with the hypo-methylation of the CpG dinucleotides in the promoter region of the H19 gene (Figure 7). This observation was clearly demonstrated through bisulfite DNA sequencing and is consistent with the concept that DNA methylation can modulate gene expression (Li et al., 1993; Feil and Khosla, 1999; Sleutels et al., 2000).

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Table 1. Summary of cDNA Microarray analyses.

Identification of genes that are differentially expressed in well-differentiated and undifferentiated NPC cells.

Genes expressed at higher levels in CNE-2 than in HK1 cells	**Genbank**	**IMAGE**
H19 mRNA, NIH_MGC_10 Homo sapiens cDNA clone	BE018809	3049170
cyclin-dependent kinase inhibitor 1C ; p57-KIP2; CDKN1C	AW612762	2957000
Protein-tyrosine kinase Flt4	AI598102	2227817
interferon, gamma-inducible protein 30	AA527870	965434
pre-B-cell leukemia transcription factor 1	AA223573	650807
thioredoxin-dependent peroxide reductase 1 (NK-enhancing factor B)	H69143	212165
Tat-interacting protein 30 kDa	AI161117	1721007
CDK2 (cell division protein kinase 2);	AW572951	2932189
BCL2-antagonist of cell death	AI245965	1871924
bcl-7B protein	AW303330	2813436
death-associated protein	W46901	324439
cyclin D3	AW316802	2827721
connective tissue growth factor precursor	AI952812	2491165
Rho GDP dissociation inhibitor (GDI) beta	AA188078	624801
cathepsin L precursor; major excreted protein (MEP)	AW572137	2750658

Genes expressed at higher levels in HK1 than in CNE-2 cells	**Genbank**	**IMAGE**
IGFBP3, Insulin-like growth factor binding protein 3	AW613832	2968906
cell cycle progression 2 protein (CPR2)	AW518910	2876723
metallothionein-Ie (hMT-Ie)	W73154	344345
cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	AI859822	2436539
monocyte chemotactic protein 3 precursor (Human)	BE046143	3126473
Human melanoma-associated antigen B3	AI954607	2473150

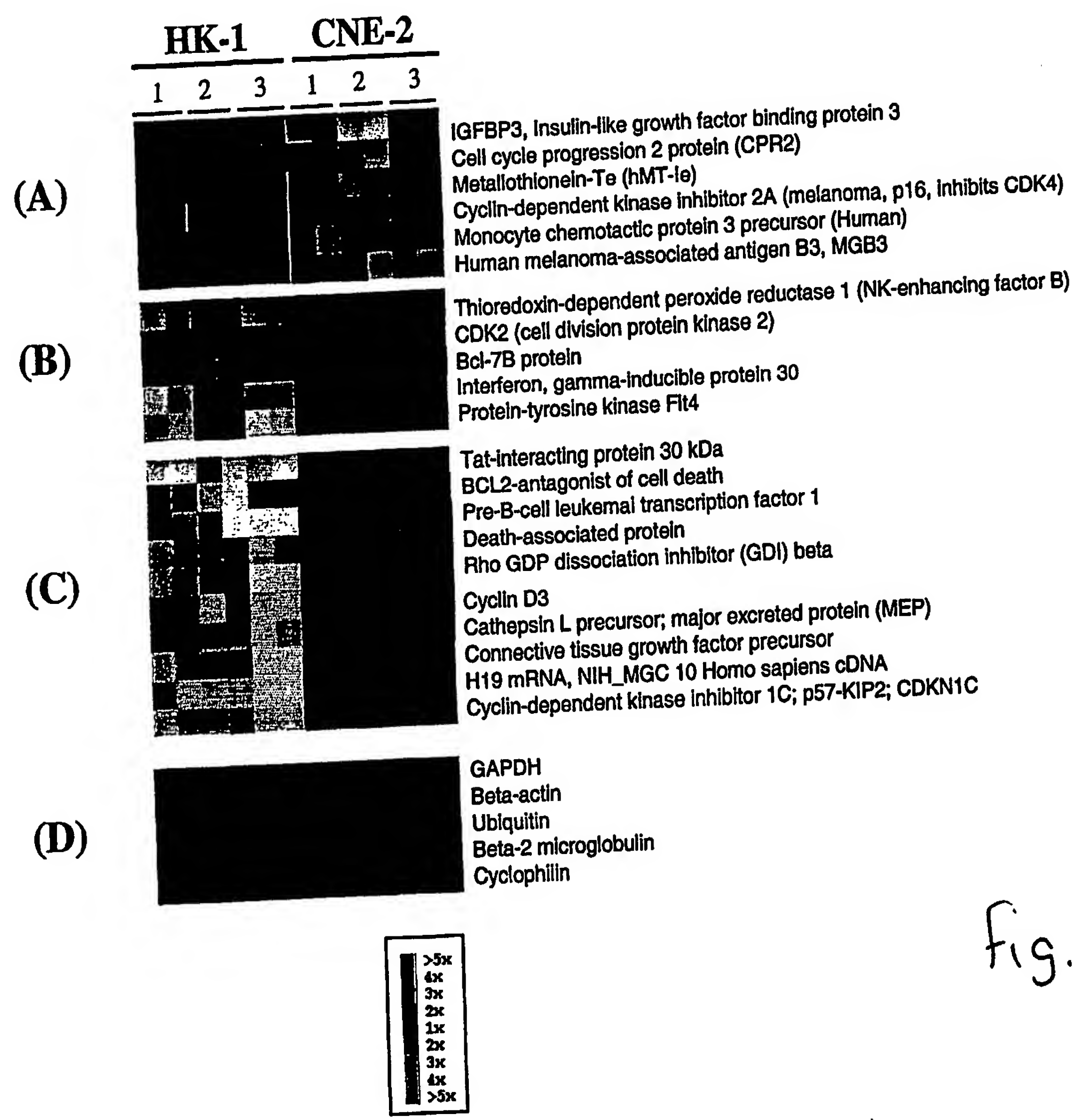

HK-1
CNE-2
1 2 3 1 2 3
(A)
IGFBP3, Insulin-like growth factor binding protein 3
Cell cycle progression 2 protein (CPR2)
Metallothionein-Te (hMT-Ie)
Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)
Monocyte chemotactic protein 3 precursor (Human)
Human melanoma-associated antigen B3, MGB3
(B)
Thioredoxin-dependent peroxide reductase 1 (NK-enhancing factor B)
CDK2 (cell division protein kinase 2)
Bcl-7B protein
Interferon, gamma-inducible protein 30
Protein-tyrosine kinase Flt4
(C)
Tat-interacting protein 30 kDa
BCL2-antagonist of cell death
Pre-B-cell leukemal transcription factor 1
Death-associated protein
Rho GDP dissociation inhibitor (GDI) beta
Cyclin D3
Cathepsin L precursor; major excreted protein (MEP)
Connective tissue growth factor precursor
H19 mRNA, NIH_MGC 10 Homo sapiens cDNA
Cyclin-dependent kinase inhibitor 1C; p57-KIP2; CDKN1C
(D)
GAPDH
Beta-actin
Ubiquitin
Beta-2 microglobulin
Cyclophilin
>5x
4x
3x
2x
1x
2x
3x
4x
>5x

Fig. 1

Figure 2

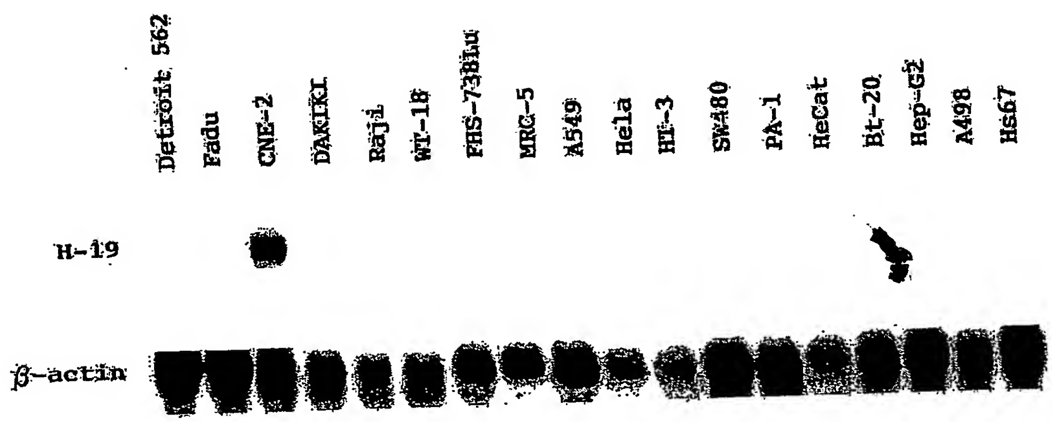

Detroit 562
Fadu
CNE-2
DAKIKI
Raji
WT-18
FHS-738Lu
MRC-5
A549
Hela
HT-3
SW480
PA-1
HeCat
Bt-20
Hep-G2
A498
Hs67
H-19
β-actin

Figure 3

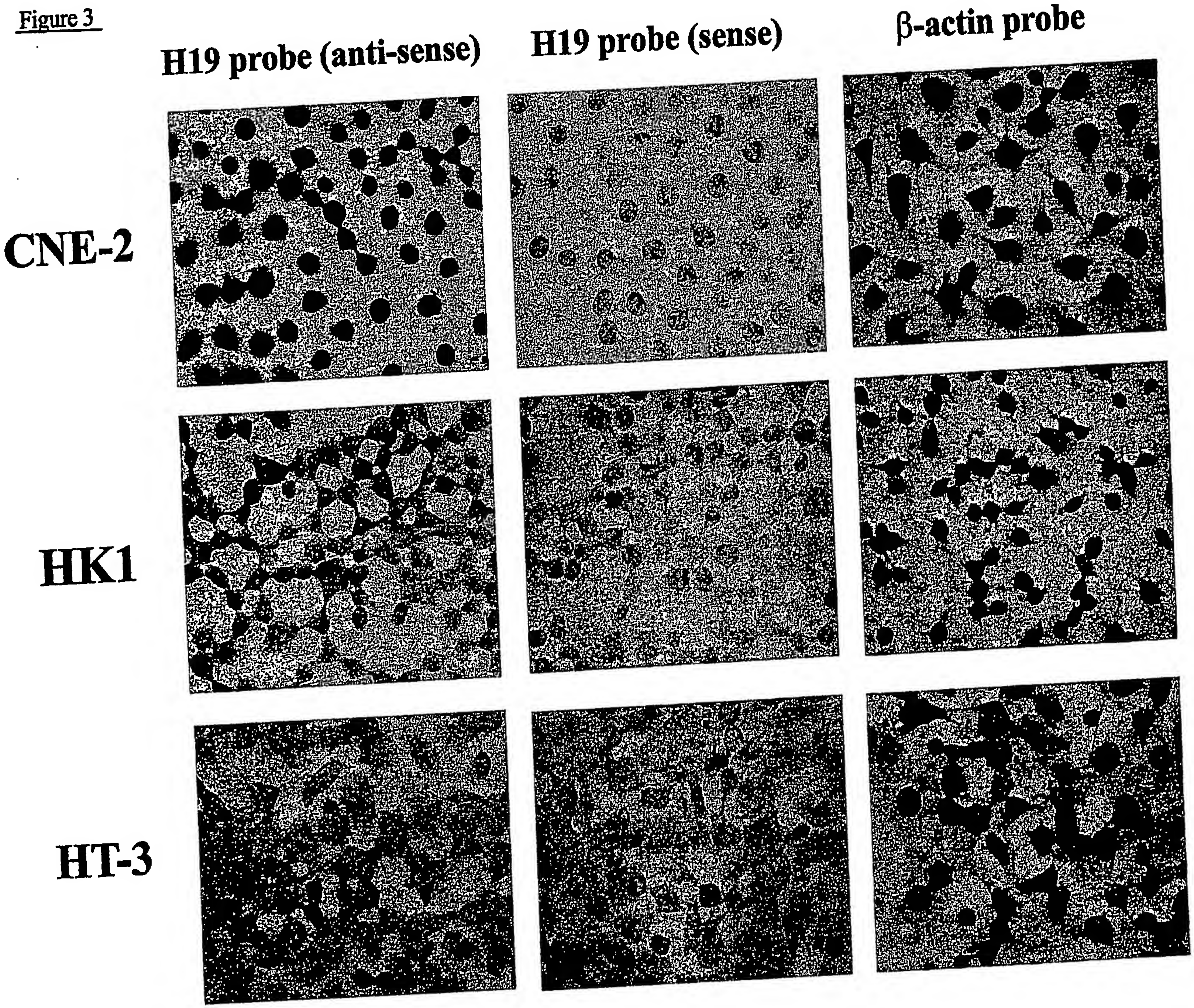

H19 probe (anti-sense)
H19 probe (sense)
β-actin probe
CNE-2
HK1
HT-3

Figure 4

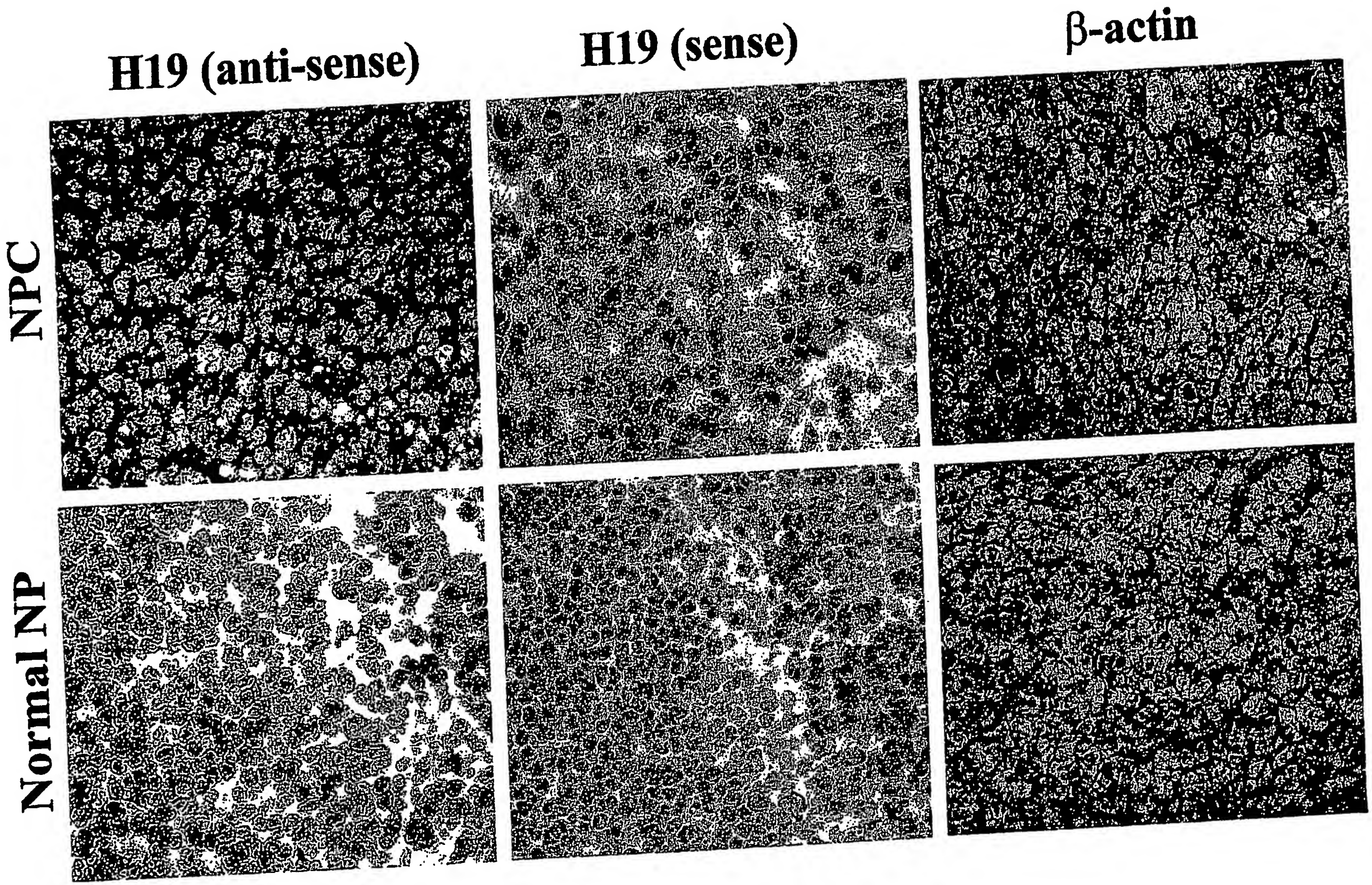


Figure 5

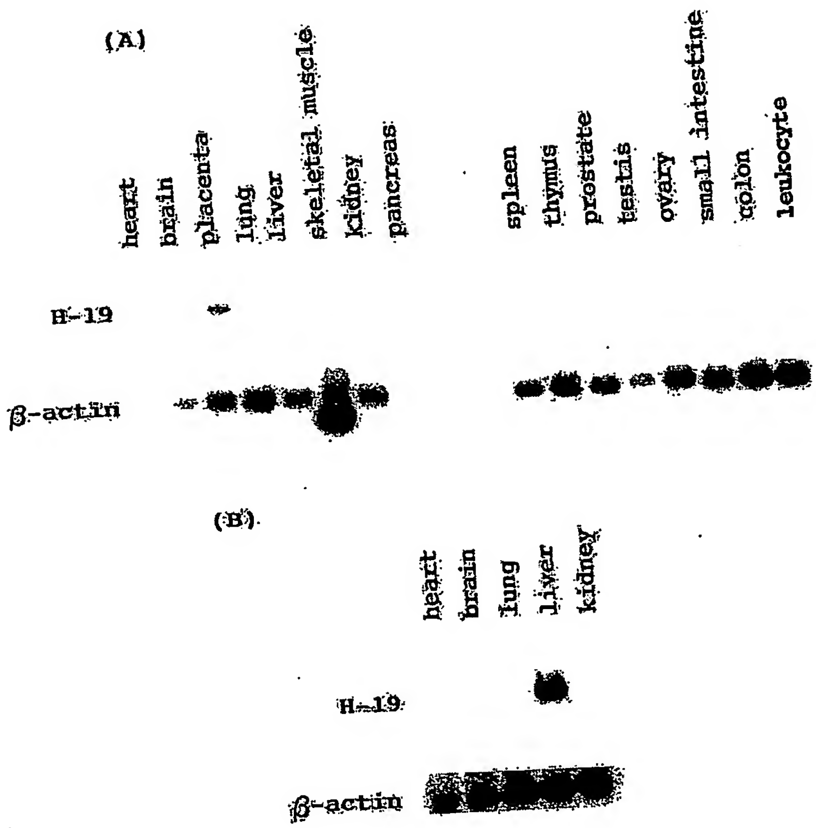

(A)
heart
brain
placenta
lung
liver
skeletal muscle
kidney
pancreas
spleen
thymus
prostate
testis
ovary
small intestine
colon
leukocyte
H-19
β-actin
(B)
heart
brain
lung
liver
kidney
H-19
β-actin

Figure 6

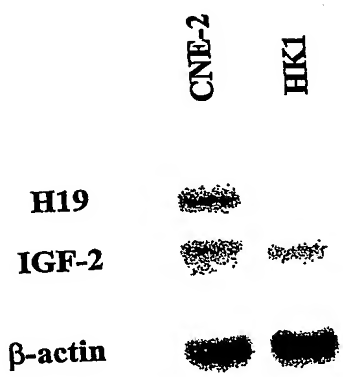
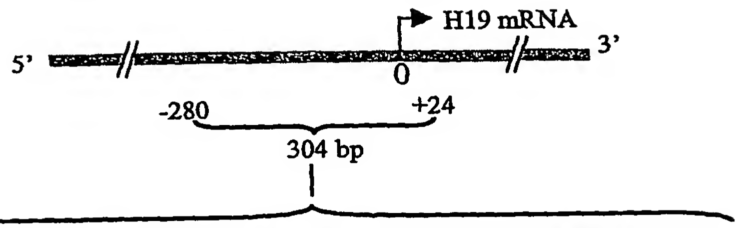


Figure 7



agacagtggtctgggagggagaggtcctggaccctgagggaggtgatggggcaatgctcagccctgtctcCGgatgccaaagga
(-209)
ggggtgCGgggaggcCGtctttggagaattccaggatgggtgctgggtgagagagaCGtgtgctggaactgtccagggCGg
(-189) (-180) (-139) (-117)
aggtgggccctgCGgggccctCGggagggccctgctctgattggcCGgcagggcaggggCGggaattctgggCGggg
(-102) (-92) (-68) (-54) (-41)
ccaccccagttagaaaaagccCGggctaggacCGaggagcagggtgagggagggggtgggat
(-15) (-4)

	Position of CpG sites / No. of clones sequenced	-209	-189	-180	-139	-117	-102	-92	-68	-54	-41	-15	-4
CNE-2	19	1 (5%)	1 (5%)	1 (5%)	0	0	0	0	0	0	0	0	0
HK1	63	50 (79%)	47 (75%)	45 (71%)	13 (21%)	45 (71%)	49 (78%)	2 (3%)	2 (3%)	5 (8%)	6 (10%)	5 (8%)	0
HK1(+AzC)	27	6 (22%)	5 (19%)	6 (22%)	4 (15%)	10 (37%)	8 (30%)	0	0	0	0	1 (4%)	0
		Number of methylated clones											

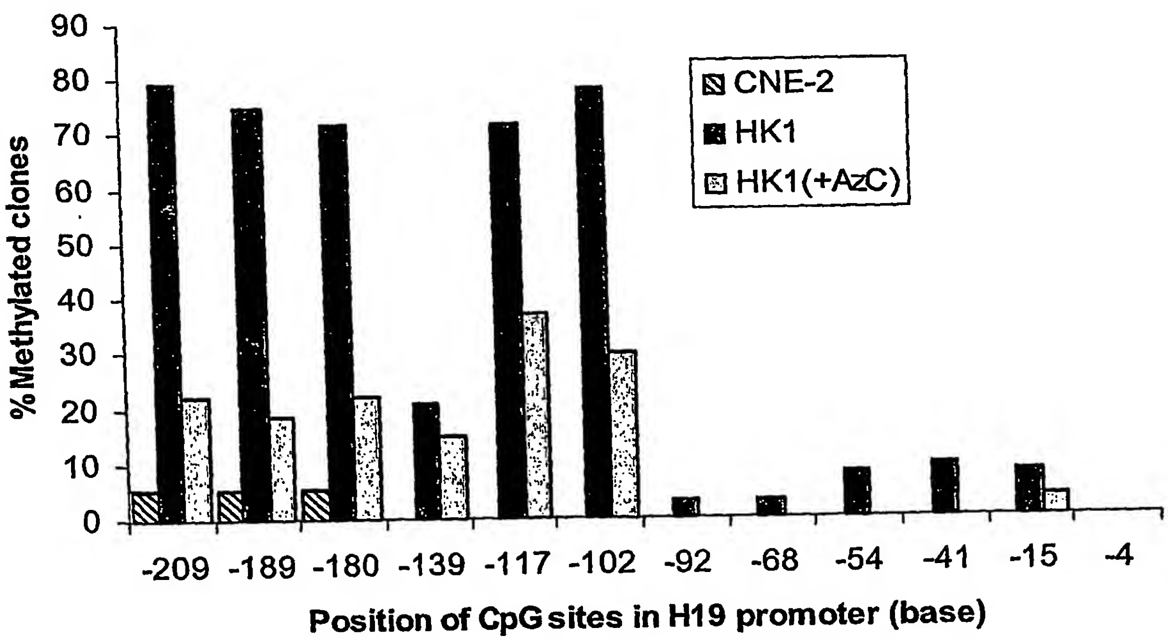


Figure 8